Liposome-Based Assay for Phospholipase C

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Phospholipase C from *Clostridium perfringens* is known to catalyze the hydrolysis of phospholipids in biological membranes. In this study, a simple and sensitive method for assaying phospholipase C was developed by using liposomes entrapping calcein as a fluorescent marker. Phospholipase C-induced lysis of liposomes was determined by measuring the fluorescence intensity of calcein released out from liposomes. Various liposomes with different compositions were prepared by reverse-phase evaporation method to investigate the effect of liposomal composition on the lytic activity of phospholipase C. The calcein-entrapping efficiency of liposomes was affected by the chain length of fatty acid in phosphatidylcholine constituting liposomes. The lytic activity of phospholipase C was the highest against liposomes prepared with eggPC. The lytic activity decreased with increasing chain length of fatty acid in phosphatidylcholine. Incorporation of cholesterol more than 20% into the liposomal bilayer inhibited the phospholipase C-induced lysis. The lysis of liposomes was more greatly increased by the addition of 10 mM of calcium. The lytic activity of phospholipase C was also affected by the surface charge of liposomes. Taken together, it was concluded that reverse-phase evaporation vesicles composed of dipalmitoylphosphatidylcholine and cholesterol in the molar ratio of 9 : 1 allowed to detect the lowest concentration of phospholipase C (0.10 μg/assay volume). This study suggested that the use of liposomes can provide a simple, sensitive and inexpensive method for assaying phospholipase C.

Introduction

Clostridium perfringens phospholipase C (alpha toxin) catalyzes the hydrolysis of glycerophosphate ester bond in phospholipids.[1] Various assay methods for phospholipase C have been reported.[2,3] The turbidimetric method has been widely used but it is, even though simple to perform, inaccurate because of the different quality of egg yolk preparations. The acid-soluble phosphorus method[2] can determine phospholipase C activity more precisely but it is laborious and does not show satisfactory detection limits. Among various methods, the use of p-nitrophenylphosphatidylcholine (NPPC), a chromogenic derivative of phosphatidylcholine (PC), offers a very simple and convenient assay method for phospholipase C.[3] Assay method measuring the hydrolysis of NPPC is known to be correlated well with that measuring the hydrolysis of PC.[4] However, this method also had some limitations such as time-consuming and expensive. Moreover, it is doubtful that all of these methods reflect the interaction of phospholipase C with substrates in biological systems because it interacts with phospholipids in cell membrane rather than a monodispersed phospholipid suspension.

Phospholipase C from *Clostridium perfringens* is also known to have lethal, hemolytic and necrotic activities in addition to its enzymatic activity.[5] Although many different bacterial phospholipase C have been isolated, few are as toxic as that from *Clostridium perfringens*. The toxicity is thought to reflect the unique feature that this protein, unlike many other bacterial phospholipase C, is readily able to interact with phospholipids in cell membranes. This interaction is thought to play an important role in the pathogenesis of *Clostridium perfringens*-mediated gas gangrene infections by promoting cell membrane disruption. Therefore, the measurement of phospholipase C-induced membrane disruption can provide an index of phospholipase C toxicity.

Liposomes, which are artificial lipid bilayers, have been extensively used as a model of membranes. Therefore, the use of liposomes for measuring the activity of phospholipase C might be a useful tool to closely mimic the interaction of phospholipase C with biological membranes.[1]

Buxton and coworkers[6] reported that the measurement of phospholipase C-induced lysis of small unilamellar vesicles (SUV) was correlated well with that of PC hydrolysis. However, there was no investigations about the factors which can affect the interaction between liposomes and phospholipase C. Moreover, the small encapsulation-volume of SUV limited the sensitivity of assay. Recently, Nagahama and coworkers[7] reported the influence of liposome composition on the activity of phospholipase C to the membrane by using multilamellar vesicles (MLV). However, MLV has not been generally used as a model membrane because its multilamellar character and many different internal volumes in each layers make the interpretation of data difficult.[8] The small encapsulation-volume and heterogeneous size of MLV are also a distinct disadvantage for applying to the phospholipase C assay.

Large unilamellar vesicles (LUV) prepared by the reverse-phase evaporation method are known to have a large encapsulation volume and single lamellar property. They can allow the encapsulation of larger amount of markers than other types of liposomes,[9] which can contribute to decreasing the detection limit of phospholipase C assay by signal amplification. Therefore, it is reported here an improved liposome-based assay for phospholipase C by using calcein-

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entrapped reverse-phase evaporation vesicles. It is also reported the effects of lipid composition, charge and cholesterol content of REVs on the lytic activity of phospholipase C.

Materials and Methods

Materials. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearylphosphatidylcholine (DSPC), stearylamine (SA), cholesterol (CHOL) and dicetylphosphate (DCP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Egg phosphatidylcholine (eggPC) was purchased from Doosan Serdary Res. Lab. (Kyungki-Do, Korea). Phospholipase C from *Clostridium perfringens* type I (4.6 units/mg solid, 6.6 units/mg protein) and type XIV (315 units/mg protein), p-nitrophenylphosphatidylcholine (NPPC) and calcein were also purchased from Sigma. Sephadex G-100 (superfine) and Sepharose CL-4B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other reagents were of reagent grade and used without further purification.

Buffers. Buffers used throughout the experiments are as follows: TBS, 10 mM Tris, 150 mM sodium chloride, pH 7.4; TBS^{2+}, 10 mM Tris, 150 mM sodium chloride, 10 mM calcium chloride, pH 7.4; PBS, 14 mM potassium phosphate, monobasic, 57 mM sodium phosphate, dibasic, 70 mM sodium chloride, pH 7.4.

Preparation and characterization of liposomes. Reverse-phase evaporation vesicles (REVs) were prepared by slight modification of the method[9] of Szoka and Papahadjopoulos. Briefly, 7.5 μmole of lipid mixture was dissolved in 1 mL of chloroform. The organic phase was removed on a rotary evaporator (Büchi RE121 Rotavapor, Büchi Co., Switzerland) at reduced pressure and the dried lipid film was dissolved in 1.5 ml of a 2 : 1 mixture of isopropyl ether and chloroform. Then, 0.5 mL of Tris buffer (10 mM Tris, pH 7.4) containing 100 mM of calcein was added dropwise and the two phases were briefly vortexed to suspend the lipid. The suspension was additionally sonicated in a bath type sonicator for 10 min at 37 °C. The organic solvent was removed under slightly reduced pressure until a clear suspension was obtained. Half milliliter of TBS was added again and the residual organic solvent was eliminated under greatly reduced pressure. The resulting liposome dispersions stood for 30 min at room temperature and were sized by extrusion through polycarbonate filters (0.1 μm pore size, Nucleopore, Costar, Cambridge, MA) assembled in a ultrafiltration cell under nitrogen pressure. The sized liposomes were eluted on Sepharose CL-4B column equilibrated with TBS buffer to remove the unencapsulated calcein. However, the liposomes containing CHOL more than 20% were adsorbed on the top of column. Instead of column purification, these liposomes were dialyzed overnight to remove free calcein. Separated liposome fractions were collected and stored in aliquots at 4 °C until use. In all experiments, the stored liposomes were diluted with buffer just prior to use.

The concentration of PC in liposomes was determined by the Stewart assay[10] with minor modifications. The encapsulation efficiency of liposomes was calculated after determining the amount of entrapped calcein by measuring the fluorescence intensity (excitation at 494 nm, emission at 520 nm) from liposomes completely lysed by Triton X-100 (final concentration, 0.2%). The size distribution of liposomes was examined by photon correlation spectroscopy. Diluted liposomes were transferred to disposable cuvettes and photon count was measured in a photon correlator at 26 °C. Data was analyzed by a laser particle analyzer (LPA-3000, Otsuka Electronics, Japan).

Effect of cholesterol inclusion on the phospholipase C-induced lysis of liposomes. Liposomes composed of DPPC and CHOL with different molar ratio (from 0 to 40%, total 7.5 μmole) were also prepared as described above. They were diluted with TBS^{2+} (total lipid concentration, 0.23 mM) and 5 μL of liposomes were incubated with 3.1 μg of phospholipase C at 37 °C in a final volume of 505 μL of TBS^{2+} buffer (pH 7.4). After one hour, the resulting mixture was diluted to 2.2 mL with the same buffer and then the fluorescence intensity of calcein released from liposomes was measured (Jasco FP-777, Japan spectroscopic Co., Japan). The percentage of lysis was calculated from equation 1.

$$\% \text{ lysis} = (F_S - F_B)/(F_T - F_B) \times 100 \quad (1)$$

where F_S is the fluorescence intensity of sample solution after lysis and F_B is the fluorescence intensity of reference solution which contains 5 μL liposome solution and buffer. F_T is the total fluorescence intensity when liposomes were lyzed completely by adding excess amount of phospholipase C or Triton X-100 (final concentration, 0.2%). All results were expressed as means ± standard deviation (S.D.).

Effect of chain length of fatty acid in PC on the phospholipase C -induced lysis of liposomes. Five microliters of liposomes composed of PC with different chain length in fatty acid (eggPC, DMPC (C14 : 0), DPPC (C16 : 0), DSPC (C18 : 0)) and cholesterol in the molar ratio of 9 : 1 was incubated with phospholipase C in a final volume of 505 μL of TBS^{2+} at varying temperatures between 4 and 59 °C. After one hour, the fluorescence intensity of calcein released from liposomes was measured and the percentage of lysis was determined according to the equation 1.

Effect of calcium on the phospholipase C-induced lysis of liposomes. Effect of calcium 10 mM in the incubation medium on the lytic activity of phospholipase C against various liposomes was also investigated by measuring the lysis of liposomes incubated with various concentrations of phospholipase C in TBS and TBS^{2+} buffer, respectively.

Effect of surface charge of liposomes on the phospholipase C-induced lysis. Liposomes composed of DPPC and CHOL (9 : 1) in the presence or absence of SA or DCP (5%) were also prepared to investigate the effect of surface charge of liposomes on the phospholipase C-induced lysis.

Application of liposome-based assay during purification of phospholipase C. Sixty miligrams of crude phospholipase C (type I) was dissolved in a minimum volume of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and applied to a column (1.5×88 cm) of Sephadex G-100. The column was eluted with the same buffer. The flow rate was 10 mL per hour and 5 mL of

each fractions were collected. The activities of protein fractions were evaluated with NPPC and DPPC-liposome, respectively. The NPPC assay was carried out in a test tube containing 200 μL of protein fractions after adding 250 μL of 40 mM NPPC, to each test tube, at 37 °C. After one hour, the reaction was terminated by adding 50 μL of 0.2 M EDTA solution (pH 7.2) and the formation of p-nitrophenol was measured spectrophotometrically at 410 nm (Beckman DU 650, Beckman Instruments Inc. Fullerton, CA, USA). On the other hand, the liposome-lytic activities of various protein fractions were measured by incubating 200 μL of protein fractions with 5 μL of liposome (DPPC: CHOL=9 : 1, equivalent to 0.21 mM of DPPC) in a final volume of 505 μL of TBS^{2+} for 30 min at 37 °C.

Results and Discussion

Various liposomes were prepared by the REV method to increase the sensitivity of the assay, since the high encapsulated volume and single lamellar property of REVs allow the encapsulation of a large amount of markers. Calcein was selected as an entrapping marker instead of macromolecules such as enzymes, considering that a larger amount of phospholipase C is required for releasing macromolecular markers from liposomes and also enzymes may make the assay procedure complicated.

At first, the lytic activity of phospholipase C on various liposomes with different compositions were investigated to optimize the assay condition.

Effect of CHOL inclusion. Liposomes composed of PC alone were relatively unstable and they showed consistent leakage of entrapped markers during storage. However, liposomes containing more than 10% of CHOL were stable for at least 6 weeks with minimal leakage of calcein at 4 °C.

With liposomes composed of PC alone, the lytic activity of phospholipase C was the highest and the inclusion of CHOL more than 20% into the PC liposomal bilayer made the liposomes more resistant to phospholipase C-induced lysis (Figure 1). It demonstrates that the lysis is a direct consequence of the hydrolysis of PC because liposomes containing higher mole% of CHOL, which have a reduced number of hydrolysis sites (mole% of PC) per liposome, were less sensitive to the lytic activity of phospholipase C. However, the percent lysis of PC liposomes was not significantly affected by the incorporation of only 10% of CHOL.

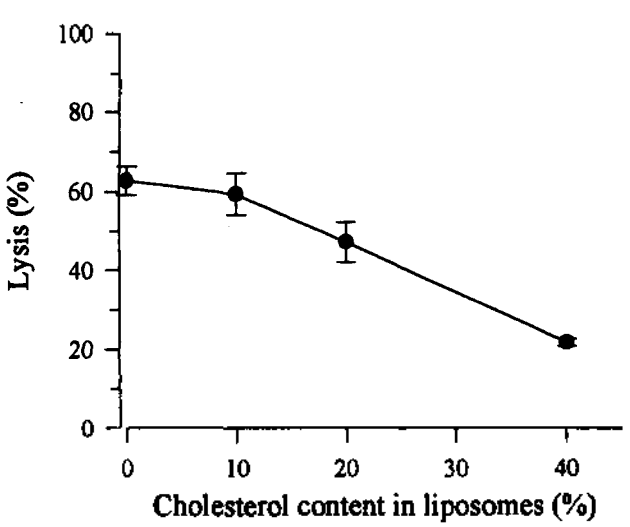

Figure 1. Effect of incorporation of cholesterol on the phospholipase C-induced lysis of liposomes. Error bars indicate SD (standard deviation) of three experiments.

In considering the assay sensitivity and liposomal stability together, liposomes containing 10% of CHOL showed the relatively good sensitivity and stability, and they were used in the subsequent study.

Chain length of fatty acid in liposomal PC. The chain length of fatty acid in PC is known to play a significant role in the phospholipase C-substrate binding. It is thought to be due to the difference of membrane fluidity.[11] Kimura,[12] however, insisted that the membrane fluidity of liposomes is not a major factor in the phospholipase C reaction, because the activity of phospholipase C was higher in the gel phases of liposomes and was almost zero at above Tc. This report is in disagreement with others.

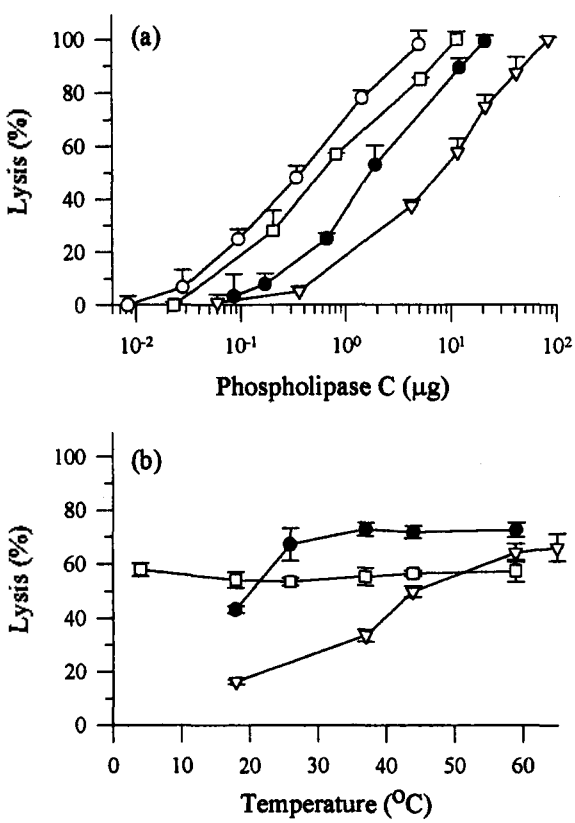

Figure 2. (a) Effect of chain length of fatty acid in liposomal phosphatidylcholine on the phospholipase C-induced lysis. (b) Temperature dependence of phospholipase C-induced lysis of liposomes. Liposomes (PC : CHOL=9 : 1) were prepared with various PC (eggPC, ○; DMPC, □; DPPC, ●; DSPC, ▽) and diluted to an appropriate concentration for fluorescence measurement (eggPC liposome (1.73 mM); DMPC liposome (0.84 mM); DPPC liposome (0.21 mM); DSPC liposome (0.20 mM)). The amount of phospholipase C for inducing lysis of liposomes is a corrected amount required for the lysis of 5 μL of corresponding liposomes (0.21 mM).

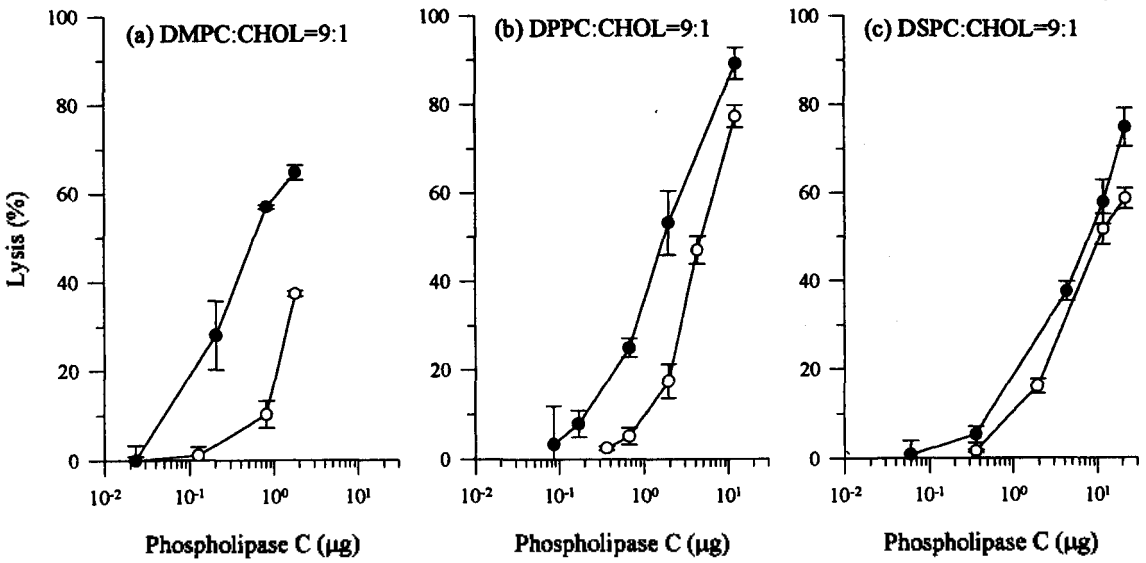

Figure 3. Effect of calcium on the phospholipase C-induced lysis of liposomes ((a) DMPC:CHOL (9:1) (b) DPPC:CHOL (9:1) (c) DSPC:CHOL (9:1)). Open and closed circles represent the percent lysis of liposomes in TBS buffer without 10 mM $CaCl_2$ and TBS^{2+} buffer with 10 mM $CaCl_2$, respectively.

In this study, The lytic activity of phospholipase C was the highest against REVs composed of eggPC and CHOL (molar ratio, 9:1). It decreased with increasing chain length of saturated fatty acid in PC constituting liposomes (DMPC (C14:0)-liposome > DPPC (C16:0)- liposome > DSPC (C18:0)-liposome) at 37 °C (Fig. 2a). The lytic activity of phospholipase C against DMPC-liposomes were not affected by the incubation temperature under current experimental conditions. However, with DPPC- and DSPC-liposomes, it tends to increase according to rising the incubation temperature and reached the plateau level at above the known phase transition temperature (Tc) of the corresponding lipids (Fig. 2b) (DPPC, 41.5 °C; DSPC, 52 °C).[13] This observation is rather in accordance with earlier report from Rie and coworkers.[11] Perhaps these discrepancies among many reports may be due to the difference in the composition, size of liposomes and the quantitation methods.

Effect of calcium. Calcium is known to play an important role in the phospholipase C reaction as a donor of positive surface charge on the micelle or by forming a relatively weak micelle-calcium preassociation complex, which then binds phospholipase C[14] or as a direct participant in an enzyme mechanism.

The phospholipase C-induced lysis of liposomes prepared with PC of shorter fatty acid chain length was more greatly increased by the addition of calcium (Fig. 3). It demonstrates that the role of calcium on the phospholipase C reaction was also dependent on the PC constituting liposomes. It may be related to the effect of calcium on the bilayer packaging condition.[13]

Although the degree of phospholipase C-induced lysis was somewhat dependent on the lipid composition, the lysis of all of these liposomes were increased by the addition of 10 mM calcium in the incubation buffer. Therefore, 10 mM of calcium were added in the buffer for the assay of phospholipase C.

Characterization of liposomes. The encapsulation efficiency of liposomes composed of PC and CHOL (9:1) increased from 0.7 to 5.9% according to the chain length of fatty acid in PC (Table 1). The encapsulation efficiencies of DPPC- and DSPC-liposomes were approximately 4 fold higher than that of DMPC-liposomes and 8 fold higher than that of eggPC-liposomes. It shows that the encapsulation efficiency of liposomes was related to the chain length of fatty acid in PC constituting liposomes. It may be due to the fact that DPPC and DSPC are in a more highly ordered state than eggPC (Tc below 0 °C) and DMPC (Tc, 23 °C) at the liposome-preparation temperature (room temperature). Incorporation of more rigid PCs during vesicle formation

Table 1. Effect of the chain length of fatty acid in phosphatidylcholine on the encapsulation efficiency of each liposome and the detection limit of assay obtained with corresponding liposomes

Composition of liposome (PC:CHOL=9:1)	Encapsulation efficiency[a] (%)	Concentration of liposome for assay[b] (mM as PC)	Detection limit of phospholipase C[c] (μg/assay volume)
egg PC:CHOL	0.7	1.73	0.18
DMPC (C14:0) :CHOL	1.4	0.84	0.15
DPPC (C16:0) :CHOL	5.5	0.21	0.10
DSPC (C18:0) :CHOL	5.9	0.20	0.33

[a] Mean values for 3 experiments. [b] Each liposomal preparations were diluted to a proper concentration prior to use in assay so that the maximum fluorescence level of calcein released from 5 μL of totally lysed liposomes could be optimally expressed within the readable range of spectrofluorometer. [c] The detection limit of assay for phospholipase C was determined by incubating with various liposomes diluted to an appropriated concentration as designated in the third column.

may help to maintain the integrity of liposomal membrane due to decreasing permeability and results in the higher encapsulation efficiency of markers.

In order to choose the proper liposomes as a substrate for the phospholipase C assay, the sensitivity to the phospholipase C-induced lysis and calcein- entrapping efficiency must be simultaneously considered since each liposome dispersion must be diluted to a proper concentration prior to use in assay so that the maximum fluorescence level of calcein released from totally lysed liposome could be optimally expressed within the readable range of spectrofluorometer. Although the lytic activity was the highest against eggPC-liposomes (Fig. 2a), the concentration of egg-PC-liposomes required for fluorescence measurement was 8.2 fold higher than that of DPPC-liposomes (1.73 mM versus 0.21 mM, Table 1).

In the consideration of both factors, the detection limit of phospholipase C assay was 0.18 μg/assay volume with eggPC liposome (lipid concentration, 8.7 nmole of PC), 0.15 μg with DMPC liposome (4.2 nmole), 0.10 μg with DPPC liposome (1.1 nmole), and 0.33 μg with DSPC liposome (1.0 nmole), respectively (Table 1). As a result, liposomes composed of DPPC and CHOL (9 : 1) detected the lowest concentration of phospholipase C and were chosen as a proper substrate for phospholipase C assay.

The size of liposomes (DPPC : CHOL=9 : 1) were approximately 240.2±69 and 175±82 nm in diameter before and after extrusion, respectively. However, the lytic activities of phospholipase C against extruded and nonextruded liposomes did not show any significant difference (data not shown). Therefore, the extrusion steps could be occasionally omitted in the subsequent experiments.

Surface charge. The activity of phospholipase C is known to be greatly influenced by the charge of phospholipid micelles.[15] Here the surface charge of liposome as a substrate was investigated for the optimization of assay. Figure 4a shows that the lowest conce ntration of phospholipase C required for inducing the lysis of negatively charged liposomes containing DCP is much lower than that required for neutral liposomes (DPPC liposome) or for positively charged liposomes containing SA (0.01 *versus* 0.09 μg). However, the concentration of phospholipase C required for 100% lysis of negatively charged liposomes was almost the same as that of neutral liposomes. The higher lysis percentage of negatively charged liposomes containing DCP in the lower concentration region of enzyme seems to be caused by calcium-induced fusion of charged liposomes not by the lytic action of phospholipase C. Therefore, the use of liposomes containing DCP may cause errors in the phospholipase C assay and its use was excluded in the subsequent study.

Standard curve for phospholipase C assay. Standard curve for phospholipase C was constructed with liposomes composed of DPPC and CHOL (9 : 1). The release of calcein from liposomes increased linearly (r=0.991) with increasing concentration of phospholipase C (Figure 4b) in the range of 0.10-22 μg per assay volume (0.21-44 μg/mL, 0.06 7-14 eggPC unit/mL, the units of enzyme are based on the supplier's (Sigma) specifications).

Application of liposome-based assay during purification of phospholipase C. The liposome-lytic activities and NPPC-hydrolytic activities of protein fractions eluted from a purification column of phospholipase C were

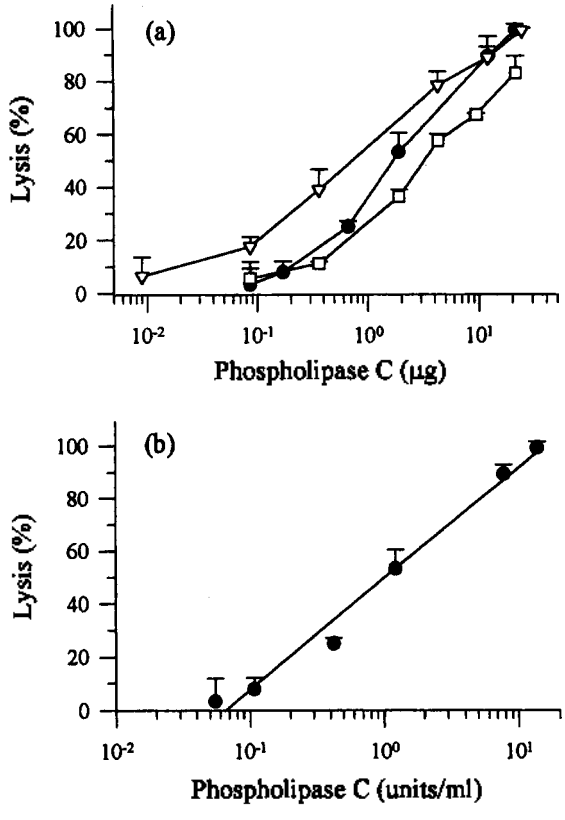

Figure 4. (a) Effect of surface charge on the lysis of liposomes (DPPC : CHOL (9 : 1), ●; DPPC : CHOL : SA (9 : 1 : 0.5), □; DPPC : CHOL : DCP (9 : 1 : 0.5), ▽) induced by phospholipase C in TBS^{2+}. (b) Representative standard curve for phospholipase C assay by using reverse-phase evaporation vesicles composed of DPPC and CHOL (9 : 1).

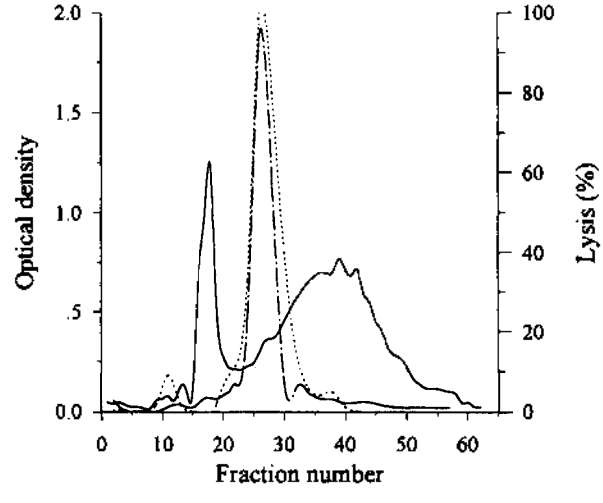

Figure 5. Application of liposome-based assay of phospholipase C during the purification step. Crude phospholipase C was eluted on a Sephadex G-100 column. The flow rate was 10 mL per hour and 5 mL of fractions were collected. The protein fractions (———) were tested for NPPC hydrolysis activity (— · —) and for liposome lytic activity (------).

compared to demonstrate the usefulness of REV as a convenient tool for assaying enzyme activity. Figure 5 shows that the protein fractions which have NPPC-hydrolytic activities also have liposome-lytic activities. In case of NPPC, at least one hour of incubation time was required for measuring the hydrolysis of NPPC but the incubation time could be shorten to less than 30 min by using liposome as a substrate. Moreover, a very small amount of liposome was required for fraction check compared to NPPC and it could lower the cost of assay (<1/100 folds).

By investigating various factors related to the phospholipase C-induced lysis of liposomes entrapping calcein, the detection limit of phospholipase C obtained by the liposome-based assay system in this study could be lowered approximately 7.5 folds than that from other system such as alkaline phosphatase-entrapped SUV[6] (0.067 versus 0.5 units/mL). Moreover, the assay was simple, sensitive and inexpensive because only a small amount of liposomes (approximately 1 nmole of PC per assay) was required for the assay.

Our finding that the lytic activity of phospholipase C is greatly affected by liposomal composition may contribute to understanding the interactions between phospholipase C and membranes in biological systems.

While this method is not specific for phospholipase C but also measures phospholipase A_2 as well as possibly phospholipase D, it can provide a simple and convenient technique to assay phospholipase C. Moreover, we have reported that the liposomal lytic activity of phospholipase C conjugated to hapten is inhibited by antibody binding on the conjugate.[16] Therefore, the investigations of liposome-lytic activity of phospholipase C should be a pre-requisite for designing a sensitive homogeneous liposome immunoassay system.

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